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GENETIC AND METABOLIC STUDIES OF APOE, PCSK9, TM6SF2 AND PNPLA3

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Genetic and metabolic studies of APOE, PCSK9 TM6SF2 and PNPLA3

THESIS FOR DOCTORAL DEGREE (PhD)

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∞ *For Diana, Christopher & Alexander* ∞

ABSTRACT

Lipids - including fatty acids (FAs), triglycerides (TGs), cholesterol and cholesterol esters (CEs) - are hydrophobic molecules involved in several important structural and mechanistic processes. Lipids are found in cellular membranes, give posture and stability to the cell, are actively participating in cellular signaling, and act as mediators in several important biological pathways. Lipids, transported between organs through lipoprotein particles, act as an important energy source. Lipids are either directly utilized in metabolic processes or, if in excess, stored in depots in the cell cytosol. However, imbalances in lipoprotein transport or cellular metabolism of the lipids may give rise to adverse cellular effects leading to metabolic disorders and cardiovascular disease (CVD). The main underlying causes for these imbalances are of dietary, environmental and genetic nature. The focus of this thesis is on the genetic causes of dyslipidemia and the roles of the proprotein convertase subtilisin/kexin type 9 (PCSK9), apolipoprotein (APO) E, transmembrane 6 superfamily member 2 (TM6SF2) and patatin-like phospholipase domain-containing protein 3 (PNPLA3) proteins.

PCSK9 is a circulating protein that influences plasma low-density lipoprotein cholesterol (LDL-C) concentration and susceptibility to cardiovascular disease. Circulating PCSK9 levels show considerable inter-individual variation, but the factors responsible for this variability are largely unknown. We analyzed circulating PCSK9 levels in 4 cohorts of healthy, middle-aged Swedes and found that PCSK9 levels varied over ~50-fold range and showed a positive relationship with plasma LDL-C concentration. Mapping of the *PCSK9* locus revealed a common polymorphism, (rs2479415, minor allele frequency (MAF) 43.9%), located ~6 kb upstream from *PCSK9*, which was independently associated with increased circulating PCSK9 levels.

It is generally assumed that the APOE concentration, in addition to the APOE ε2/ε3/ε4 genotype, influences plasma lipoprotein levels, but a functional genetic variant influencing the plasma APOE concentration has not been identified. In a genome-wide association (GWA) study, we observed that the *APOE* locus was the only genetic locus showing robust associations with the plasma APOE concentration. Fine-mapping of the *APOE* locus showed that rs769446 (-427T/C) in the *APOE* promoter is independently associated with the plasma APOE concentration. The minor allele of rs769446 is associated with increased *APOE* mRNA levels ($p = 0.015$) as analyzed in 199 human liver samples. Transient transfection studies and electrophoretic mobility shift assays in human hepatoma HepG2

cells corroborated the role of rs769446 in transcriptional regulation of *APOE*.

TM6SF2, a gene with unknown function, encodes a protein of 351 amino acids with 7-10 predicted transmembrane domains. It is located on chromosome 19-12, a locus associated to the plasma TG concentration and hepatic lipid content. Gene expression studies in human liver samples demonstrated that *TM6SF2* was the putative causal gene for this association. Subcellular localization studies showed that TM6SF2 is localized in the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment of human liver cells. Functional studies evaluating the secretion of TG-rich lipoproteins (TRLs) and lipid droplet (LD) content in Huh7 and HepG2 cells showed that *TM6SF2* inhibition was associated with reduced secretion of TRLs and increased cellular triglyceride concentration and LD content, whereas *TM6SF2* overexpression lead to reduced liver cell steatosis.

The *PNPLA3* gene variant I148M is an important marker of human non-alcoholic fatty liver disease (NAFLD), but the physiological function of PNPLA3 in liver fat metabolism remains unclear. We therefore analyzed *PNPLA* mRNA levels in human and mouse tissues and evaluated the effect of small interfering RNA (siRNA) silencing of *PNPLA3* on TG metabolism in human Huh7 and HepG2 hepatoma cells. Although *PNPLA3* had the highest expression level of all PNPLA family members in 91 human liver samples, *PNPLA3* silencing in Huh7 and HepG2 cells was not associated with changes in TRL secretion, cellular triglyceride content and the rate of triglyceride synthesis.

LIST OF PUBLICATIONS

I. Chernogubova E, Strawbridge R, **Mahdessian H**, Mälarstig A, Krapivner S, Gigante B, Hellénius ML, de Faire U, Franco-Cereceda A, Syvänen AC, Troutt JS, Konrad RJ, Eriksson P, Hamsten A, van 't Hooft FM. **Common and low-frequency genetic variants in the PCSK9 locus influence circulating PCSK9 levels.**
Arterioscler Thromb Vasc Biol. 2012;32:1526-1534

II. Mannila MN, **Mahdessian H**, Franco-Cereceda A, Eggertsen G, de Faire U, Syvänen AC, Eriksson P, Hamsten A, van 't Hooft FM. **Identification of a functional apolipoprotein E promoter polymorphism regulating plasma apolipoprotein E concentration.**
Arterioscler Thromb Vasc Biol. 2013;33:1063-1069

III. **Mahdessian H**, Taxiarchis A, Popov S, Silveira A, Franco-Cereceda A, Hamsten A, Eriksson P, van 't Hooft F. **TM6SF2 is a regulator of liver fat metabolism influencing triglyceride secretion and hepatic lipid droplet content.**
Proc Natl Acad Sci *U S A*. 2014;111:8913-8918

IV. **Mahdessian H**, Taxiarchis A, Franco-Cereceda A, Eriksson P, Hamsten, van ’t Hooft F. **Role of patatin-like phospholipase domain-containing protein 3 (PNPLA3) in the regulation of triglyceride metabolism in human Huh7 hepatoma cells.** Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ABCA	ATP-binding cassette transporter A
ACAT	Acetyl-CoA acetyltransferase
APO	Apolipoprotein
CE	Cholesterol ester
CETP	Cholesteryl-ester transfer protein
CHD	Coronary heart disease
CM	Chylomicron
COP	Coatamer protein
CVA	Cerebrovascular disease
CVD	Cardiovascular disease
DG	Diacylglyceride
DGAT	Diacylglycerol O-acyltransferase
eQTL	Expression quantitative trait locus
ER	Endoplasmic reticulum
FA	Fatty acid
GPAT	Glycerol 3-phosphate acyltransferases
GWA	Genome-wide association
HDL	High-density lipoprotein
HSL	Hormone-sensitive lipase
IDL	Intermediate-density lipoprotein
LCAT	Lecithin cholesterol acyl transferase
LD	Lipid droplet
LDL-C	Low-density lipoprotein cholesterol
LDLR	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
LRP	LDLR-like protein
MG	Monoacylglyceride
MAF	Minor allele frequency

MGLL	Monoacylglyceride lipase
MOGAT	Monoacylglycerol O-acyltransferase
MTTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver disease
PAD	Peripheral arterial disease
PCSK9	Proprotein convertase subtilisin/kexin type 9
PLIN	Perilipin
PNPLA	Patatin-like phospholipase domain-containing protein
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SR	Scavenger receptor
T2D	Type 2 diabetes
TG	Triglyceride
TRL	TG-rich lipoprotein
TM6SF2	Transmembrane 6 super family member 2
VLDL	Very low density lipoprotein

1 INTRODUCTION

Lipids are molecules with high structural diversity that are involved in many tightly controlled physiological processes. The lipid diversity within cells, organelles, organs and species is highly conserved and important throughout evolution.[1] To maintain homeostasis, triglycerides (TGs) should comprise 20-25% of total energy intake, the ratio of polyunsaturated to saturated fatty acids (FAs) should exceed 1.0, and that total serum cholesterol levels should be below 5 mmol/L.[2] However, since the agricultural and especially the industrial revolution, we have experienced a conflict between our current diet/environment and our ancient genome.[3] The shift towards increased consumption of saturated FAs, salt and carbohydrates has disturbed lipid homeostasis and resulted in an unprecedented increase of people suffering and dying from metabolic and cardiovascular disease (CVD).

The causes of dyslipidemia are not only dietary or environmental. In nature it has been shown that genetic predisposition plays a vital role in lipid homeostasis and in the susceptibility to metabolic disorders and CVD.[4] Monogenic forms of dyslipidemia, such as familial hypercholesterolemia have long been known to be associated with CVD.[5] These disorders are caused by a single mutation within a gene that raises total cholesterol levels over 7 mmol/L. In complex diseases, the interaction of several low-impact genetic variants together with effects of dietary and environmental factors give rise to disease. Defining the genetic contribution in multigenic lipidemia and its implications in disease is therefore much more difficult. However, technological developments in the field of genome sequencing and genotyping have provided new insights in our understanding of these genetic variations. Together with qualitative functional studies this has shed new light on the mechanisms of dyslipidemia and their implication in diseases. This thesis focuses on defining the physiological roles of four genes involved in lipid metabolism.

1.1 LIPOPROTEIN METABOLISM

Cholesterol ester (CE) and TG molecules are transported to different organs for energy usage and storage. However, due to their non-polar carbon tail they are not compatible to freely flow via the blood or lymphatic vessels. This transport is mediated through the packaging of CEs and TGs in polar lipoprotein particles. Lipoprotein particles are composed of a core of non-polar lipids and a surface of polar proteins and phopholipids. They are synthesized in the liver and the intestine. A family of surface proteins called

apolipoproteins (APO) serves as signal proteins in lipid metabolism. The size and nature of these lipoproteins varies according to their core lipid density and the nature of their surface proteins. The different lipoproteins are in order of size from largest to smallest (Figure 1): chylomicrons (CM), very-low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL).[6] Once in circulation, these particles are highly dynamic. They undergo size and conformation changes through hydrolysis and transfer reactions of their core lipid components and of their apolipoproteins. Lipoproteins are catabolized in the liver, kidney and the peripheral tissue via receptor-mediated mechanisms.[7]

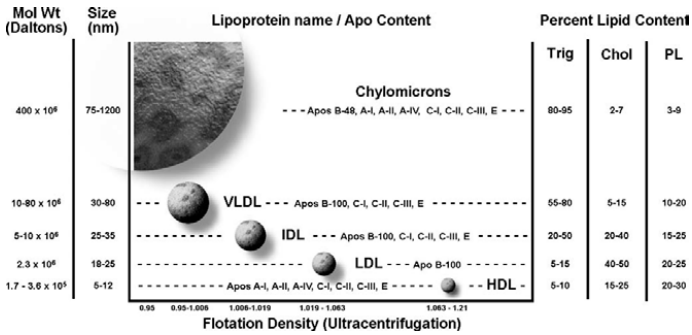

Figure 1. Classification of lipoproteins based on floatation density.
Obtained from JM Saland, H. Pediatric Nephrology 2007;22: 1095-1112.

1.1.1 Exogenous lipoprotein metabolism

Dietary lipids are digested in the gut into glycerol and long chain FAs by bile salts and pancreatic lipases. Enterocytes absorb the glycerol molecules and FAs to resynthesize TGs, which are then assembled, with the help of the lipid transfer protein, microsomal triglyceride transfer protein (MTTP), in the endoplasmic reticulum (ER) and packaged together with APOB48 and APOA4 into pre-CMs. In the Golgi, the CMs gain more TGs and CEs and also acquire additional apolipoproteins. [8-12]

1.1.2 Endogenous lipoprotein metabolism

Apart from the uptake of lipids from the diet, the liver has the ability to synthesize its own TGs and CEs. The origins of the FAs used for TG synthesis are i) de novo FA synthesis from acetyl-CoA, a process known as lipogenesis ii) hydrolysis of the plasma lipoproteins cleared by the liver iii) uptake of circulating FAs derived from the hydrolysis of adipose tissue lipids. These TGs enter the cytosolic TG pool in the liver and are then mobilized together with CEs and APOB100 protein to assemble VLDL particles. In the ER lumen of the hepatocytes,

APOB100 is co-translationally lipidated by MTTP, giving rise to pre-VLDL. Once this particle is sufficiently lipidated, it is retained in the ER, continuing lipidation to give rise to VLDL2. The VLDL2 particles in the ER are either converted to larger VLDL1 in the Golgi through further addition of TGs or secreted as VLDL2 into the lumen and ultimately the blood. The mechanism by which the cells secrete VLDL1 or VLDL2 is not yet fully understood.[8-12]

1.1.3 Lipolysis and uptake of lipoproteins and its remnants

CM secreted into circulation acquire APOE, APOC2 and APOC3 from HDL. Lipoprotein Lipase (LPL), activated by APOC2, hydrolyzes the TGs of the CMs into FA, which diffuse through the capillaries into muscle or skeletal cells. The lipolysis of TG gives rise to smaller particles called CM remnants, which are removed from the circulation by the liver primarily through LDL-receptor (LDLR) and LDLR-like protein 1 (LRP-1) with APOE as ligand. Once in the circulation, VLDL acquires APOC2. TGs in the VLDLs, similar to that of CMs, are hydrolyzed by LPL on the luminal surface of the capillary endothelium in muscle and adipose tissue. As the TGs are released, the VLDL shrinks in size and forms IDL particles. IDL has two fates; it is either removed from the circulation by a process mediated by APOE or undergoes further lipolysis by LPL losing APOE, apoC and more FAs, alternatively yielding LDL. Plasma LDL clearance occurs primarily via LDLR-mediated endocytosis in the liver and peripheral cells, with APOB100 as a ligand. The affinity of this binding is less than the APOE/LDLR affinity, resulting in a long half-life of the cholesterol-rich LDL in the circulation.[13-17]

1.1.4 HDL and reverse cholesterol transport

HDLs are lipoprotein that traffic cholesterol. They are synthesized in the liver and the intestine, lipidated on the surfaces of these organs with the help of ATP-binding cassette transporter A1 (ABCA1). APOA1 and APOA2 are the main lipoproteins on the surface of HDL molecules. Newly synthesized HDL particles obtain APOE and APOCs in an exchange process between HDL and APOB containing particles. In addition, HDLs acquire cholesterol from other lipoproteins and organ tissues during their time in the circulation. Most notable of these tissues is the arterial wall, hence reducing the cholesterol deposit burden. The continued esterification of free cholesterol into CE by the enzyme lecithin-cholesterol acyltransferase (LCAT) renders the maturation of the HDL molecule. Eventually, HDL particles empty their cholesterol load in the liver with the help of scavenger receptors (SRs) by a process called the direct cholesterol-transport pathway. HDLs are also able to exchange their cholesterol

molecules with TGs of VLDLs and CMs, a process facilitated by cholesteryl-ester transfer protein (CETP). The transport of these cholesterol particles by LDL to the liver is known as the indirect cholesterol transport pathway. These processes are modulated by several enzymes and cell membrane transporters that lipidate or de-lipidate HDL. According to this model, increased numbers of APOB containing VLDL or LDL result in the transfer of excess TGs to HDL in exchange for CE molecules, thereby depleting the HDL particle from cholesterol molecules.[8-12, 17-18]

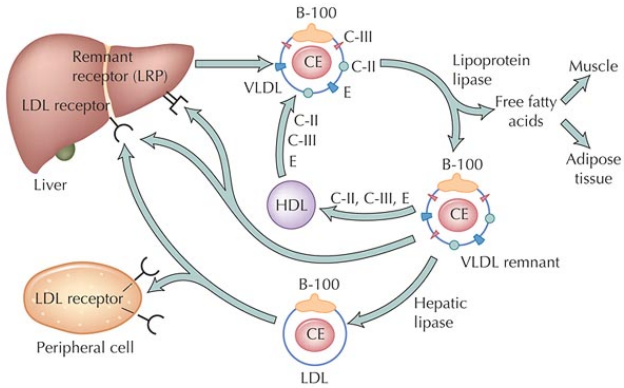

Figure 2. Schematic representation of lipoprotein metabolism

1.2 INTRACELLULAR LIPID METABOLISM

1.2.1 Lipid droplets

Until a decade ago, lipid droplets (LDs) were considered to be inert depots of fat found in mammalian adipose tissues. It is now known that LDs are found in all mammalian cells. LDs are dynamic organelles involved in lipid metabolism and also participate in response to ER stress, protein glycosylation and pathogen infection.[19-22] LDs are composed of a mass of neutral lipids, predominantly CEs and TGs, surrounded by a phospholipid monolayer. The lipid monolayer harbors different proteins that are involved in the regulation and metabolism of lipids.[19, 23-25] LDs are localized in the cytosol of most cells, but their quantity and size varies across different cell-types. The two main cell-types that contain LDs are adipocytes and hepatocytes. Adipocytes contain one large LD in the cytoplasm (up to 100μm in diameter) whereas hepatocytes contain a large number of LDs of relatively smaller size (2-10μm in diameter).

1.2.2 Proteins involved in LD metabolism

Neutral lipids are formed from the transfer of FAs to lipid alcohols. Several ER membrane-bound enzymes utilize FAs to catalyze this step. For example, diacylglycerol acyltransferase (DGAT) enzymes utilize FA and diacylglycerol (DG) to form TGs. Similarly; FA is condensed with cholesterol to produce CEs. The FA substrates for the neutral lipids are either supplied through i) lipogenesis, where acetyl-CoA acts as a precursor to a group of FA synthetases such as acetyl-CoA acetyltransferases (ACATs), glycerol 3-phosphate acyltransferases (GPATs) and Lipins or from ii) plasma uptake and esterification of free FAs after lipoprotein degradation and transferring them to form more complex neutral lipids with the help of monoacylglycerol O-acyltransferases (MOGATs) and DGATs.[19]

1.2.3 LD formation

The exact mechanism by which LDs are formed is unknown. A reason for this is the small size of the nascent LDs and the difficulties of available technologies to detect such small molecules in a time-lapse fashion. However, the general consensus for LD origins is the ER membrane. Three models for nascent LD biogenesis are proposed: (Figure 3)

a) "Lensing model": Neutral lipids are deposited between the ER-lumen and cytosolic leaflets of the ER membrane, producing a bulging on the cytosolic leaflet size, which dissociates into an LD particle once a certain size is reached. Membrane proteins are dissociated with the cytosolic leaflet composing the LD membrane.
b) Bicelle formation: Neutral lipids are deposited in similar fashion as the "lensing model", but both leaflets of the ER membrane constitute the LD membrane as they are excised from the ER once a certain size is reached.
c) Vesicle formation: The leaflets are rearranged forming a vesicle, followed by active TG incorporation.[23-28]

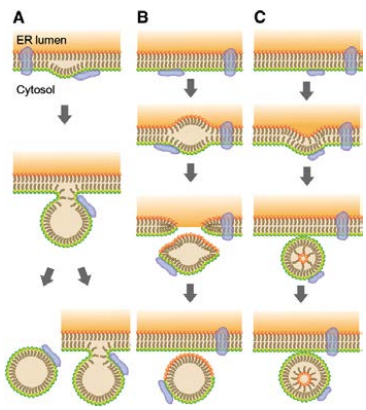

Figure 3. LD formation according to
A) Lensing model, where the neutral lipid core bulges from the cytosolic membrane of the ER after reaching a critical size.
B) Bicelle formation, neutral lipids fill the ER membrane and both membranes of the ER are excized to form the outer LD membrane.
C) Vesicle formation through rearrangement of the innermost membrane and active TG incorporation to form the LD.

Obtained from Van der Klei IJ. et. Al. Genetics. Jan;193(1):1-50. (2013).

1.2.4 LD growth

The mechanism for LD growth is not yet fully understood, but several mechanisms have been proposed. One proposed mechanism involves the synthesis of TGs at the surface of LDs. Surface phospholipids are removed from the LDs by the coatamer protein (COP) 1 machinery, increasing surface tension of the LDs facilitating the interaction with the ER-membrane, establishing an ER-LD membrane-bridge. This bridge facilitates the unidirectional relocation of TG synthesis enzymes from the ER to the LD to locally produce TGs, leading to the growth of the LD. Another proposed mechanism of LD growth is through permeation, where neutral lipids are actively transferred from a smaller to a larger LD. Fat specific protein 27 is an enzyme facilitating this transfer in adipocytes. A third proposed mechanism of LD growth is through fusion. Here, two LDs with different surface tensions interact and form one larger LD.[29-33]

1.2.5 LD lipolysis

Lipid homeostasis in organs is crucial to prevent diseases such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), obesity and various forms of lipodystrophy. FAs that are not directly oxidized for energy production are packaged as TGs in LDs for later use. How, when and at what rate TGs and CEs are hydrolyzed differs depending on the organ, cell type, milieu and nutritional need. LD-TGs are hydrolyzed in all cells through the activity of lipases recruited to the LD surface by LD surface proteins. The best known intracellular lipases are adipose triglyceride lipase (ATGL, now called PNPLA2), hormone-sensitive lipase (HSL) and monoacylglyceride lipase (MGLL). These enzymes act on TG (PNPLA2), (DG) (HSL) or MG (MGLL) to release FAs that are either used in energy production or incorporated with other lipid molecules. It was shown that LDs smaller in size are generally degraded faster than larger LDs. This is probably due to the higher lipolytic activity of lipases on smaller LD surfaces where these proteins have a denser surface-to-volume ratio. There are however conditions in which larger LDs are degraded quicker than smaller LDs. This is explained by conditions rendering differential localization of regulatory surface proteins, i.e. Perilipin1 (PLIN1). It was recently shown that PNPLA3, a protein with homology to PNPLA2, might also have intracellular lipolytic properties in liver and adipocytes. A mutated PNPLA3 (I148M) was observed to promote TG accumulation and hepatic steatosis.[34-38]

1.2.6 LD heterogeneity

LDs are ubiquitously expressed dynamic organelles in the body. They are of different sizes and protein composition. These differences are thought to be the result of parallel actions of lipogenesis, growth, lipolysis and the unique composition and density of the surface proteins in each specific LD. The biological significance of this heterogeneity lies in maintaining the homeostasis of intracellular lipid levels by controlling the FA flow, but it is not clear how the size affects the metabolic fate of LDs.[30-35]

1.3 PATHOPHYSIOLOGY OF DYSLIPIDEMIA IN CARDIOVASCULAR DISEASE

1.3.1 Introduction

CVD is the leading cause of death worldwide. An estimated of 17.3 million people died of CVD in 2008. In Sweden, 1.4 million people live with the disease and 40% of the total deaths are due to CVD. The estimated costs of CVD for the year 2013 exceeded 60 billion SEK. CVD comprises a group of disorders of the heart and the blood vessels. The most important of these disorders are: coronary heart disease (CHD), involving the blood vessels supplying the heart muscle; cerebrovascular disease (CVA), involving the blood vessels supplying the brain; peripheral arterial disease (PAD), involving the blood vessels supplying the arms and legs; deep vein thrombosis and pulmonary embolism, the consequence of blood clots in the leg veins, which can dislodge and move to the heart and lungs.[39-40]

1.3.2 CVD and risk factors

CVD is associated to several risk factors. These include a history of CVD, male gender, age, ethnicity, socio-economic status, family history of CVD, smoking, alcohol consumption, type 1 and 2 diabetes mellitus, hypertension, obesity and disturbances of the plasma lipid profile. Some of these factors, such as age and gender are fixed and cannot be reversed; however other factors are adjustable and/or preventable. Intervention and improvements of these factors reduces the CVD burden. For example, healthy diet and physical activity reduce obesity, control blood pressure and improve the lipid profile; leading to a reduction of CVD risk. [39-40]

1.3.3 Plasma lipid profile

Controlling the plasma concentration of lipoproteins is currently the most effective way of preventing CVD. Specifically, drugs aimed at reducing plasma LDL-C concentrations (Statins) have substantially reduced CVD deaths and prolonged lives. A lipid profile is

considered atherogenic when one or several of the following measurements is observed; elevated total cholesterol (> 5.0 mmol/L), elevated LDL-cholesterol (>3.0 mmol/L), elevated TG (>2.0 mmol/L), and low concentration of HDL-C (<1.0 mmol/L). [41-46]

1.3.4 Plasma lipids and disease

Therapies focusing on LDL-C reduction with statins have substantially reduced the CVD burden and saved many lives over the past four decades. However, the benefits of LDL-C lowering have shown to be not as efficient in patients suffering from extremely high LDL-C levels or other more complicated forms of dyslipidemias. There are also a substantial number of hypercholesterolemic patients experiencing adverse side effects from statins. Alternative LDL-C lowering therapies, such as PCSK9 inhibitors, are being clinically tested for these individuals. More recently, focus in treating abnormal lipid levels has broadened. Increasing number of patients suffering from Type 2 diabetes (T2D), obesity and insulin resistance are all characterized by elevated plasma TG levels reflecting in an increase concentration of remnant VLDL or CMs in the circulation. Patients with metabolic disorders such as insulin resistance are characterized by unbalanced FA turnover in the liver and in the adipose tissues. This causes an overproduction of larger VLDL particles leading to increased plasma TG levels, and low HDL levels and the generation of small, dense, CE-depleted LDL.[45-55]

1.3.5 Intracellular lipids and disease

The plasma TG concentration is closely linked to TG metabolism in the liver. Excessive accumulation of TGs in the liver leads to hepatic steatosis, a condition associated with adverse metabolic consequences, including insulin resistance and dyslipidemia, two important components of the metabolic syndrome. NAFLD is the most common form of hepatic steatosis in Western countries. This disease can develop from simple hepatic steatosis to irreversible cirrhosis.[56-61]

1.4 GENOME WIDE ASSOCIATION (GWA) STUDIES

A GWA study constitutes a biologically unbiased scanning of the genome of a population with the aims to find genomic loci that are associated with disease or intermediary phenotypes. It is an advanced form of linkage-mapping, a method developed to identify the cause of Mendelian disorders. To date, more than 2000 single nucleotide polymorphisms (SNPs) across all diseases have been discovered by GWA studies.[61-65]

1.5 MAPPING OF EXPRESSION QUANTITATIVE TRAIT LOCI

Expression of quantitative trait loci (eQTL) mapping is a genomics approach to infer directly or indirectly the functional effect of genetic variants on gene expressions. Advances in technological platforms have allowed simultaneous mapping of the genome and the transcriptome in search for differences in genetic variation affecting gene expression. eQTL has been a crucial complement to quantitative trait locus (QTL) mapping, which is the mapping of genetic variation to a certain trait or phenotype. The ultimate goal of eQTL analyses is to find a genetic variation that either effects the expression of that particular gene (cis-eQTL) or the expression of a distant gene (trans-eQTL). eQTL analyses has also facilitated on understanding of the intricate relationship of gene networks with respect to causality to complex diseases.[66-68]

2 AIMS

The overarching aim of the thesis is to functionally characterize genes involved in the regulation of hepatic TG metabolism.

The specific aims of these studies were:

- To identify the genetic factors responsible for inter-individual differences of circulating plasma PCSK9 levels **(paper I)**
- To uncover the functional genetic variant(s) influencing the plasma APOE concentration **(paper II)**
- To identify and functionally characterize the gene in the 'NCAN region' that is responsible for the association with plasma TG concentration and liver TG content **(paper III)**
- To determine the role of PNPLA3 in the regulation of TG metabolism in human Huh7 hepatoma cells **(paper IV)**

3 MATERIAL AND METHODS

3.1 STUDY COHORTS

Biobanks and databases originating from 5 population-based studies of middle-aged subjects from the greater Stockholm area were used.

- POLCA consists of 649 healthy 50-year-old men, free from CHD, who had been recruited at random using the population registry.
- OLIVIA comprises 340 men and 340 women with a similar age distribution (33–80 years), recruited according to the same principles for inclusion in the PROCARDIS program.
- CORONA consists of 750 healthy men, free from CHD, aged between 40 and 65 years (with 30 subjects for every age group), who had been recruited at random using the population registry.
- 60YO is a population-based, cross-sectional cohort of a total of 4232 60-year-old men and women. Subjects on statin medication, with manifest diabetes mellitus, of non-European descent, or with incomplete genotype data were excluded from all analyses.

  Overall, a total of 624 subjects from POLCA, 591 subjects from OLIVIA, 719 subjects from CORONA, and 3788 subjects from 60YO were evaluated.
- ASAP is a cohort of 600 consecutive patients undergoing elective surgery due to aortic valve disease. Liver biopsy was taken from 210 of these subjects.

These studies were performed with standardized protocols, approved by the Ethics Committee of the Karolinska Institute. All subjects were included after informed or signed consent.

3.2 GENOTYPING

Genotyping was performed using the Illumina (San Diego, CA) Infinium II Human 1M Beadchip for the OLIVIA cohort. The Illumina Infinium 610K quad Beadchip was used for the POLCA cohort. Illumina Human 610W-Quad Beadchip was used for the ASAP cohort at Uppsala University, Sweden.

The GenomeStudio software from Illumina was used for genotype calling and quality control.

Imputation was carried out on the quality-controlled genotypes of the combined POLCA-OLIVIA data set.
All Taqman genotyping assays were conducted in 384-well format, measured in the 7900HT fast real-time polymerase chain reaction system (Applied Biosystems), and analyzed with the auto-analysis program.

3.3 GENE EXPRESSION AND eQTL ANALYSIS

Gene expression was analyzed in all liver samples using the Affymetrix GeneChip Human exon 1.0 ST array (Affymetrix, Santa Clara, CA). Blood was collected from all patients and genotyping was performed using Taqman assays (Applied Biosystems, Life Technologies, Carlsbad, CA). Standard plasma lipid analysis was performed with automated commercial assays. All protocols were approved by the ethics committee of the Karolinska Institutet and all subjects signed an informed and written consent.

3.4 CELL CULTURE CONDITIONS AND TRANSFECTION STUDIES

Human hepatoma HepG2 and Huh7 cells were obtained from the American Type Culture Collection (HB-8065; Manassas, VA) and the Health Science Research Resources Bank (cell no. JCRB0403; Osaka, Japan), respectively. Cells were grown in Dulbecco's Modified Eagle's Medium (Gibco, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin. Lipofectamine 2000 (Invitrogen, Life Technologies, Carlsbad, CA) was used as transfection reagent. siRNA oligonucleotides were purchased from Ambion (Life Technologies, Carlsbad, CA). *Trans*IT®-2020 Transfection Reagent (Mirus Bio LLC, Madison, WI) was used as transfection reagent for the overexpression studies. C-terminal GFP-tagged or FP635-tagged full-length human TM6SF2 and CALR probes were obtained from OriGene (Rockville, MD).

3.5 CELL FIXATION AND STAINING

HepG2 and Huh7 cells were cultured in 4-well chamber slides. Fixation was performed with 3% Paraformaldehyde. All washing steps were performed with room temperature PBS.

- For the antibody staining, cells were permeabilized for 45 minutes. Primary staining was performed with PDI, ERGIC53 or GIANTIN mouse monoclonal antibodies

(Enzo Life Sciences, Farmingdale, NY). Secondary staining was performed with goat anti-mouse Alexa Fluor 488 secondary antibody (Invitrogen, Life Technologies, Carlsbad, CA).

- For the lipid-droplet analysis, cells were stained with 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene (BODIPY493/503, Molecular Probes, Life Technologies, Carlsbad, CA).

All cells were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA).

3.6 CONFOCAL MICROSCOPY

Images were obtained using a Leica SP5 confocal microscope, equipped with a 63×1.4 lens and diode and argon lasers. All images were generated using ImageJ software (http://rsb.info.nih.gov/ij/). Lipid-droplet area was quantified in each chamber slide using the Particle analysis Plugin of the ImageJ software. The number and area of the cells in each field were determined using the ImageJ cell-counter and particle analysis Plugin.

3.7 BIOCHEMICAL ASSAYS

Venous blood samples were obtained after an overnight fast. Plasma and serum intended for the biobank were aliquoted and stored at −70°C until analysis.

Standard lipid analyses were performed with automated commercial assays, and APOB was determined by an immunoturbidometric assay.

Plasma insulin concentration was measured by ELISA based on a monoclonal antibody (DAKO Ltd for POLCA and DakoCytomation Ltd for OLIVIA, Dako, Ely, Cambridgeshire, UK).

Plasma concentrations of C-reactive protein (high-sensitivity range) and fibrinogen were determined by immunoturbidometric assays in the BN system (Dade Behring, Liederbach, Germany).

Serum PCSK9 concentration was determined as described by Alborn et al.13

The TM6SF2 antibody used in the Western blot experiments was purchased from Abnova (Heidelberg, Germany). Gene expression in the cell-culture experiments was quantified by real-time quantitative PCR using Taqman assays (Applied Biosystems, Life Technologies, Carlsbad, CA); RPLP0 was used as standard.

TG secretion was quantified following 24 hour incubation of cells with ^{14}C glycerol

(PerkinElmer, Waltham, MA) at a final concentration of 2.85 mCi/mL. Lipids were phase separated by hydrophobicity phase separation and the upper lipophilic layer containing the TGs was separated using thin layer chromatography.
The APOB in the cell-culture medium was quantified by ELISA (ALerCHEK, Springvale, ME).
Cellular TG and protein concentrations were measured with assays supplied by Biovision (Milpitas, CA) and Thermo Fisher Scientific (Waltham, MA).

3.8 STATISTICAL ANALYSIS

The distribution of continuous variables in groups is expressed as mean ± SD or ± SEM.
Differences in continuous variables between groups were tested by Student´s *t* test.
The transcript units and the plasma triglyceride levels were logarithmically transformed for all other statistical analysis.
Multiple linear regression was used to test the association between mRNA expression and the SNP coded by the number of major alleles, while ANOVA analysis was used to test the association between mRNA expression and the SNP genotypes.
The Rcoloc values in the colocalization studies were calculated using the colocalization Plugin of the ImageJ software.
Statistical analysis was performed in GraphPad Prism, PLINK and STATISTICA (STATsoft, Tulsa, OK). Calculation of linkage disequilibrium and identification of regions with high linkage disequilibrium were performed in Haploview 4.2.

4 RESULTS AND DISCUSSION

4.1 COMMON AND LOW-FREQUENCY GENETIC VARIANTS IN THE PCSK9 LOCUS INFLUENCE CIRCULATING PCSK9 LEVELS

4.1.1 Aim

Several studies have shown that circulating PCSK9 levels exhibit up to 50-fold variation in healthy, middle-aged subjects. It is therefore generally assumed that genetic components play an important role in the inter-individual variation in circulating PCSK9 levels. However, only one common genetic variant with a marked effect on circulating PCSK9 levels has thus far been identified, the rs11591147 SNP resulting in the *PCSK9*-R46L variant. The minor allele of rs11591147 has a frequency of only 1-2% in healthy Caucasians and is associated with a marked reduction in plasma LDL-C concentration. However, the rs11591147 SNP explains less than 1% of the overall variation in circulating PCSK9 level, suggesting that there are additional genetic variants influencing circulating PCSK9. Here, we used the GWA study approach to identify novel genetic variants contributing to the variation in plasma PCSK9 levels and correspondingly LDL-C concentrations [71-73,76,80].

4.1.2 Results

We performed a GWA study using a discovery cohort of 1215 subjects and a replication cohort of 4507 subjects. All cohorts were composed of healthy, middle-aged subjects and none of the subjects were using lipid-lowering medication. In line with previous reported studies, a 50-fold variation of circulating PCSK9 levels was observed in all cohorts. Moreover, consistent positive relationships between the circulating PCSK9 levels and the plasma LDL-C and TG concentrations were observed. However, no SNPs were identified in the GWA study that showed a genome-wide statistical significant relationship with circulating PCSK9 level. Further analysis for the *PCSK9*-R46L variant in the discovery and replication cohorts demonstrated that the minor allele of the rs11591147 SNP is indeed associated with markedly reduced plasma PCSK9 levels and correspondingly LDL-C concentration. We decided therefore to focus on the *PCSK9* locus and search in this genomic region for additional, independent SNPs associated with circulating PCSK9 levels. Dense SNP mapping of the *PCSK9* locus was performed in the 1215 subjects from the discovery cohort. This resulted in the identification of 8 SNPs associated to circulating PCSK9 levels. Subsequent genotyping of the replication cohort identified 2 SNPs with genome-wide statistical significant relationships with the circulating PCSK9 levels: the

previously reported rs11591147 (*PCSK9*-R46L) with a MAF 0.02 and a common polymorphism rs2479415 with MAF 0.44. The two SNPs were independently related to circulation PCSK9 levels. However, rs11591147 showed a strong relationship with the plasma LDL-C concentration, while only a weak relationship between rs2479415 and the plasma LDL-C concentration was observed.

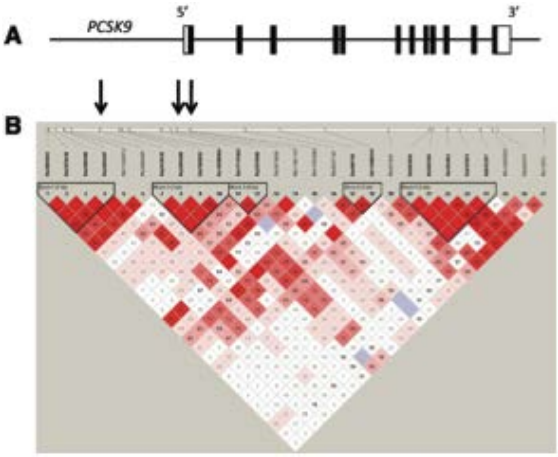

Figure 4. A) *PCSK9* gene structure. B) Linkage-disequilibrium plot of SNPs in the *PCSK9* gene locus from 1215 subjects. Colors and values reflect R^2 measures of linkage disequilibrium. Arrows indicate the positions of the rs2479415 and rs11591147 SNPs.

As shown in Figure 4, the position of rs2479415 SNP is 6kb upstream of the *PCSK9* transcriptional start site. This encouraged us to analyze the relationship between rs2479415 and hepatic *PCSK9* mRNA levels. As shown in Figure 5, a graded relationship between the rs2479415 genotypes and *PCSK9* mRNA levels was observed. In contrast, no association was observed between rs11591147 and hepatic *PCSK9* mRNA levels.

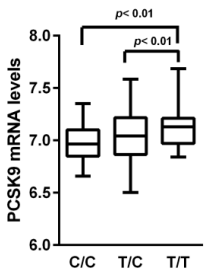

Figure 5. Relationship between rs247915 genotype and statin-adjusted PCSK9 mRNA levels in 199 liver samples. P-values are from Student's *t* test.

4.1.3 Discussion

In this study we identified the rs2479415 SNP in the *PCSK9* promoter associated with circulating PCSK9 levels. This relationship is independent from the association between the rs11591147 SNP and circulating PCSK9 levels. Indeed, the minor allele of the rs2479415 SNP is associated with increased PCSK9 levels, while the minor allele of the rs11591147

SNP is associated with decreased PCSK9 levels. Moreover, a relationship between the minor allele of the rs2479415 SNP and the *PCSK9* mRNA concentration was observed, while no relationship between the rs11591147 SNP and *PCSK9* mRNA levels were found. It is therefore tempting to speculate that the rs2479415 SNP in the *PCSK9* promoter influences the rate of transcription of *PCSK9*, ultimately leading to differences in circulating PCSK9 levels. Although the current data is in agreement with this hypothesis, there remain a number of unresolved questions. For example, our fine-mapping analysis is by current standards not really comprehensive. Indeed, a systematic analysis of all SNPs with a MAF>1% in a larger region around the *PCSK9* gene in a considerably larger population is required to identify all SNPs with an association with circulating PCSK9 levels independent from the *PCSK9*-R46L mutation. This type of comprehensive fine-mapping is currently feasible and should lead to the identification of one or more additional SNPs with independent relationships to circulating PCSK9 levels. Indeed, we refrained from further functional analysis, as described for example for the SNP in the *APOE* promoter in Study II, while we were not certain that the rs2479415 SNP is indeed the 'top' SNP associated with circulating PCSK9 levels.

The GWA study performed in this study did not lead to the identification of a new genetic locus related to circulating PCSK9 levels. Nevertheless, the same discovery cohort identified a robust genetic locus associated with the plasma APOE concentration (see Study II). This underlines some of the difficulties and uncertainties with the use of the GWA study approach for the identification of genetic loci for quantitative phenotypes. It is important that there is sufficient genetic contribution to the variation of the phenotype analyzed in the GWA study. As will be reported elsewhere, we found - using a classical twin-study design - that genetic factors accounted for ~36% of the variation in circulating PCSK9 levels. Another factor that may influence the success of the GWA study is the genetic coverage of the genome by the SNP array. In general, commercially available arrays are designed to capture the relationships between phenotype and common SNPs and are seldom able to identify relationships with SNPs with MAF <5%. An alternative approach to capture relatively rare SNPs in GWA studies is to evaluate the extremes of the distribution patterns of the phenotype, in this case subjects with very high or extremely low PCSK9 levels. However, it is generally agreed that an increase in the size of the study population, thereby increasing the power of the GWA study, is the single, most significant factor determining the success of the GWA study. By current standards, a discovery cohort of 1215 subjects in generally insufficient to identify novel genetic loci using the GWA study approach [71-81].

4.2 IDENTIFICATION OF A FUNCTIONAL APOLIPOPROTEIN E PROMOTER POLYMORPHISM REGULATING PLASMA APOLIPOPROTEIN E CONCENTRATION.

4.2.1 Aim

APOE is a surface protein found on TRLs that act as a ligand for clearing of these lipoproteins from circulation through binding to LDLR. APOE has three known alleles ε2, ε3 and ε4 coding for the APOE2, APOE3 and APOE4 isoproteins, respectively. The APOE isoproteins interact with different affinities with the LDLR. APOE2 has lower affinity for LDLR as compared to the common APOE3 isoprotein, while there is some evidence that APOE4 has a slightly higher affinity for LDLR as compared to APOE3. These differences in affinities for LDLR influence the rate of removal of remnant lipoproteins, but it is not clear if this phenomenon accounts for the whole spectrum of lipoprotein changes associated with the *APOE* locus.

The association between the APOE isoforms and the plasma lipoprotein levels has been studied in considerable detail and it appears that the APOE isoforms are related to changes in the plasma cholesterol, TG, as well as APOE concentrations. It is generally assumed that the APOE isoforms are the primary genetic and functional determinants of the observed relationships with plasma LDL-C concentration Nevertheless, it remains uncertain if there are other genetics variants in the *APOE* locus that contribute to the regulation of the plasma lipoprotein levels, specifically in relation to the plasma TG and APOE concentrations. Here, we analyzed the impact of genetic variants in the *APOE* locus on the plasma APOE concentration.[82-87]

4.2.2 Results

We performed a GWA study of the plasma APOE concentration in 1766 subjects (Figure 6). A highly significant relationship was observed between the *APOE* locus and the plasma APOE concentration. However, no additional genetic loci with a relationship to the plasma APOE concentration were found. We therefore decided to concentrate on a further exploration of the *APOE* locus in an extended cohort of 2687 subjects. Using a dense map of SNPs in the *APOE* locus we identified four SNPs with a significantly association to plasma APOE concentration. The rs769446 SNP, located in the proximal promoter of *APOE*, was the only SNP that was independently associated to plasma APOE concentration, in addition the rs429358 and rs7412 SNPs which are the polymorphisms responsible for the *APOE* ε2/ε3/ε4 genotypes.

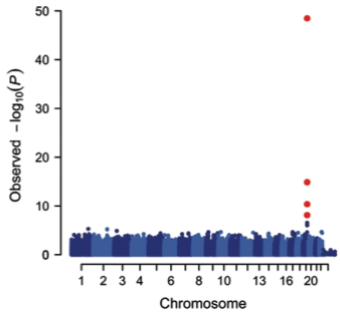

Figure 6. Manhattan plot. *P* values for association with plasma apolipoprotein E (APOE) concentration. rs769446, rs405509, rs429358, and rs7412 shown in red achieved genome-wide significance.

The relationship between rs769446 and the *APOE* mRNA expression was analyzed in 199 liver samples. It was found that the minor allele of the rs769446 SNP was associated with increased hepatic *APOE* mRNA levels (Figure 7). Of note, the minor allele of the rs769446 SNP was also associated with increased plasma APOE concentration, indicating that the rs769446 SNP in the *APOE* promoter influences the rate of transcription of *APOE*, ultimately leading to an increased plasma APOE concentration. Functional studies were performed in HepG2 cells to substantiate this hypothesis. As shown in Figure 7 the C-allele of the rs769446 SNP showed a higher basal rate of transcription as compared to the common T-allele (Figure 7B). Electromobility shift assay analysis demonstrated quantitative differences in the binding of nuclear factor(s) to the different alleles of the rs769446 SNP. Overall, these observations are compatible with the notion that the rs769446 SNP influences the binding of nuclear factor(s) to the *APOE* promoter, thereby influencing the rate of transcription of *APOE*, ultimately leading to an increased plasma APOE concentration of carriers of the minor allele of the rs769446 SNP.

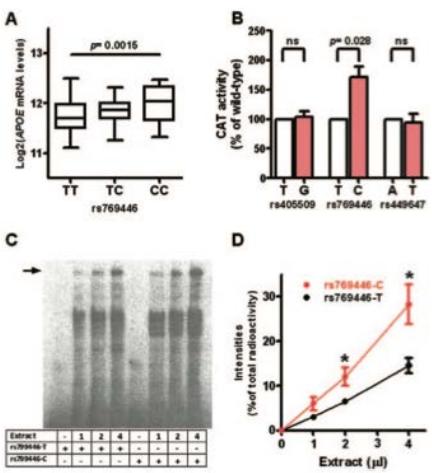

Figure 7. A) Graded association between rs769446 and plasma APOE levels in 199 subjects. Increased basal transcription rate of the rs769446-C allele as compared with the rs769446-T allele shown in B, and by electrophoretic mobility shift assay showing a distinct difference in binding of nuclear factors between 30-bp fragments containing either the rs769446-C site or the rs769446-T site. D) Quantitative analysis of protein–DNA complex indicated with **arrow** in **C** demonstrating significant differences between the rs769446-C and the rs769446-T fragments.

The availability of a SNP with a relationship to the APOE concentration independent from the *APOE* genotype creates the opportunity to test the effect of APOE concentration on plasma lipoprotein levels. To this end we analyzed the relationships between the rs769446 and plasma lipoprotein concentrations in subjects with the *APOE* ε3/ε3 genotype, thereby excluding the effects of the APOE genotype on lipoprotein levels. As shown in Table 1, no evidence was found for an association between the rs769446 genotype and plasma lipoprotein concentrations.

Table 1. Associations between rs769446 genotype and the plasma lipoprotein concentrations in subjects with the *APOE* ε3/ε3 genotype.

	60YO (n=1648)		POLCA-OLIVIA-CORONA (n=1039)		Combined (n=2687)	
Trait	β (SE)	*P* value	β (SE)	*P* value	β (SE)	*P* value
Triglycerides	0.000 (0.031)	0.993	-0.046 (0.043)	0.293	-0.018 (0.025)	0.490
Cholesterol	-0.001 (0.011)	0.900	0.002 (0.014)	0.908	0.000 (0.009)	0.972
LDL-C	-0.009 (0.016)	0.574	0.002 (0.020)	0.899	-0.005 (0.012)	0.710
HDL-C	0.025 (0.016)	0.115	0.003 (0.022)	0.882	0.017 (0.013)	0.195
APOB	-0.008 (0.014)	0.583	0.006 (0.018)	0.749	-0.003 (0.011)	0.788

4.2.3 Discussion

In this study we identified and functionally characterized the rs769446 SNP in the *APOE* promoter. An important characteristic of this SNP is the associated to the plasma APOE concentration. This relationship is independent from the APOE genotype. This feature of the rs769446 SNP makes it possible to evaluate the association between the plasma APOE concentration and the plasma lipoprotein concentrations. We performed this analysis in two large cohorts of subjects with the *APOE* ε3/ε3 genotype, thereby excluding the impact of the APOE genotype on lipoprotein levels. However, no evidence was found for an effect of the rs769446 SNP on plasma lipoprotein concentrations, suggesting that variation of the plasma APOE concentration has no effect on the plasma concentrations of the major lipoprotein classes. This observation is surprising, while it was generally assumed that the APOE concentration, in addition to the APOE genotype, was a determinant of plasma lipoprotein concentrations. Indeed, case-control studies had suggested that the plasma APOE concentration influences the risk for CHD, presumably as a consequence of the effect of the APOE level on plasma lipoprotein concentration. However, the absence of an effect of genetic variation of the plasma APOE concentration on the plasma lipoprotein levels, as reported in this study, makes it unlikely that the APOE concentration influences the risk of CHD. Nevertheless, this conclusion remains to be tested experimentally.

It has generally been assumed that the APOE genotype is exclusively responsible for the relationships between the *APOE* locus and the plasma lipoprotein concentrations. The identification of the rs769446 SNP in the *APOE* promoter as a functional SNP with an

independent relationship with the plasma APOE concentration thus provides evidence for a role of addition SNPs in the *APOE* locus in the regulation of plasma lipoprotein concentrations. As outlined above, there is evidence that the APOE genotype plays a major role in the regulation of the plasma LDL-C concentration. However, there is no convincing experimental data substantiating a role of the APOE genotype in the regulation of the plasma TG concentration. Indeed, the *APOE* gene is located in close proximity to *APOC1*, a member of the APOC-family with roles in the metabolism of TRLs. This raises the possibility that other SNPs in the *APOE* locus effect the metabolic function of APOC1, thereby influencing the plasma TG concentration independent from the *APOE* genotype. This hypothesis can be tested experimentally by performing a fine-mapping analysis of the *APOE* locus in a large cohort of healthy subjects and evaluating the relationship with plasma TG concentration. [82-87]

4.3 TM6SF2 IS A REGULATOR OF LIVER FAT METABOLISM INFLUENCING TRIGLYCERIDE SECRETION AND HEPATIC LIPID DROPLET CONTENT.

4.3.1 Aim

Several GWA studies have identified a locus on chromosome 19p12, sometimes termed NCAN locus, associated with the plasma TG and LDL-C concentrations. More recently, the same genetic locus was found to be associated with hepatic lipid content in healthy human subjects. However, at least 19 different genes are present in the chromosome 19p12 locus and the gene responsible for the reported associations has not been identified. The aim of this study was to identify the causal gene and to characterize the functional role of this gene in TG metabolism.[88-89]

4.3.2 Results

When performing genome wide expression analysis in relation to different plasma lipid levels in 206 liver samples, we observed a strong correlation between *TM6SF2*, a gene with then unknown function, and circulating TGs levels (Figure 8). This gene was located on the 19p12 locus, which was previously shown to being associated to hepatic and plasma TG levels. The lead SNP in these reports was the rs10401969. We observed that the carriers of the minor rs10401969 allele had lower *TM6SF2* mRNA levels, indicating a role of the polymorphism on both mRNA levels and circulating TGs. We also observed, in accordance with recent observations, that TM6SF2 was expressed in the liver and the small intestine; organs of importance regarding synthesis and secretion of TRLs.

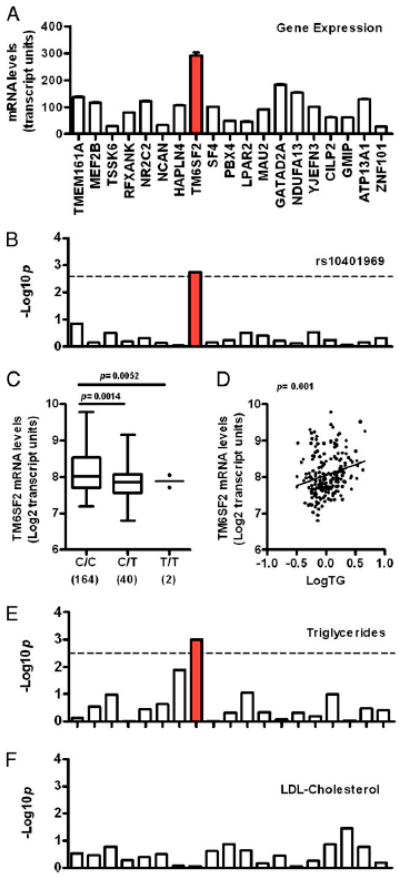

Figure.8 A) Hepatic mRNA levels of the 19 genes in the 19p12 locus. B) –Log10p-value plot of rs10401969 association to the mRNA levels of the 19 genes in the 19p12 locus. C) Association of the rs10401969 genotypes to hepatic *TM6SF2* mRNA levels. D) Hepatic *TM6SF2* mRNA correlation to plasma TG levels. E) –Log10p-value plot of mRNA levels of all the genes in the 19p12 locus to plasma TG levels. F) –Log10p-value plot of mRNA levels of all the genes in the 19p12 locus to plasma cholesterol levels

Nothing about TM6SF2 other than its sequence homology to TM6SF1 was known to that date. The nomenclature suggested it to be a transmembrane protein. Analysis of the sequence in ProtScale and other alignment tools showed 7-10 transmembrane rigions within the gene. To localize TM6SF2 we transfected Huh7 cellls with GFP tagged plasmids and observed the protein to be expressed in the ER (Figure 9).

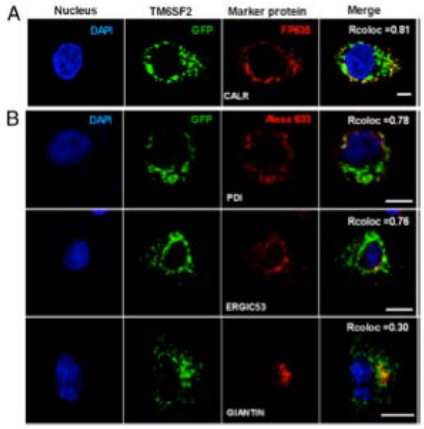

Figure 9. A) Localization of TM6SF2-GFP (green) and CALR-FP635 (red) proteins in Huh7 cells. B) Localization of TM6SF2-GFP (green) with Alexa633-tagged PDI, ERGIC53 and GIANTIN antibodies in Huh7 cells.

To characterize the functionality of the TM6SF2, we performed a series of transient transfection experiments in Huh7 and HepG2 cells using TM6SF2 siRNA and TM6SF2-tagged plasmids. We observed an increase in intracellular TGs and a reduction of secreted TGs when silencing the TM6SF2 (Figure 10).

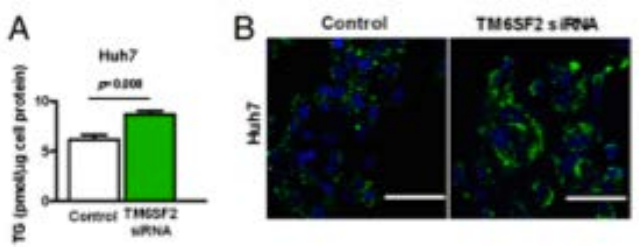

Figure 10. Accumulation of TGs in Huh7 cells after TM6SF2 inhibition measured with a) biochemical assay and B) confocal microscopy (green represents TG staining).

Interestingly, TM6SF2 siRNA reduced the expression of secreted APOB100 also and showed to reduce the expression of genes involved in TG synthesis (Figure 11).

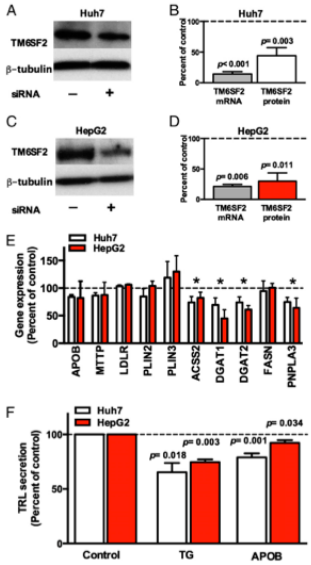

Figure 11. A & C) Western blot analysis in Huh7 and HepG2 cells 48-hours post TM6SF2 inhibition. B & D) Protein quantification of the western blot bands in A & C. E) Expression of genes involved in hepatic lipid metabolism 48-hours post TM6SF2 inhibition F) Secreted TG and APOB levels 48-hours post TM6SF2 inhibition in Huh7 and HepG2 cells.

Reversely, we observed increased accumulation of TGs in cells overexpressing the TM6SF2 protein. We could not measure the secreted TRLs in the overexpressed experiments probably due to a low net effect of the expressed TM6SF2 protein on in-vitro TG levels (Figure 12).

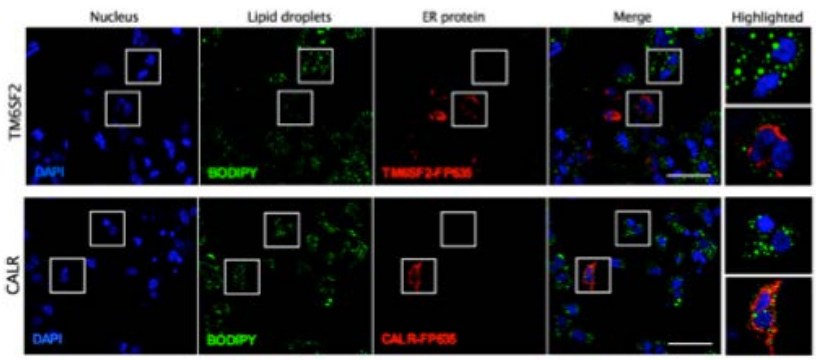

Figure 12. Huh7 cells transfected with *TM6SF2*-FP635 (above red) or *CALR*-FP635 (below red). Green represents TGs in LDs. Higher TG accumulation observed in cells overexpressing the TM6SF2 protein compared to the cells not overexpressing TM6SF2.

4.3.3 Discussion

We found that this protein was an ER membrane protein with 7-10 trans-membrane regions. In Huh7 and HepG2 cells, the inhibition of TM6SF2 showed dual functionality; we observed increased hepatic TG levels as well as LD size and content reducing secreted TGs. Reversely, the increased expression of the protein had opposing intracellular effects to inhibition; LD content was reduced in cells overexpressing the TM6SF2 protein.

Recently, two other studies reported similar results in mice models. Knockdown of Tm6sf2 or transient TM6SF2 overexpression in mice alters serum lipid profiles and influences hepatic TG content. However the functional role of TM6SF2 in regulating intracellular and secreted TG levels remains unclear.

TM6SF2 is solely expressed in the intestine and the liver in humans, organs of importance regarding TRL secretion. Inhibition of the protein although having reduced effects on both secreted TG and APOB levels, the magnitude of this reduction varies, therefore suggesting less incorporation of TGs in VLDL particles. *TM6SF2* inhibition also reduces the expression of TG synthesis genes. We therefore conclude that TM6SF2 probably primarily affects VLDL synthesis and secondarily affects TG accumulation and LD formation. Our views slightly deviate from the conclusions of the other groups who believe TM6SF2 is involved in the regulation of VLDL secretion and not synthesis. However, all of these groups accept the impairment of the expression of the gene to causing intracellular TG accumulation.[88-93]

4.4 ROLE OF PATATIN-LIKE PHOSPHOLIPASE DOMAIN-CONTAINING PROTEIN 3 (PNPLA3) IN THE REGULATION OF TRIGLYCERIDE METABOLISM IN HUMAN HUH7 HEPATOMA CELLS

4.4.1 Aim

The *PNPLA3* gene variant I148M is an important marker of human non-alcoholic fatty liver disease (NAFLD), but the physiological function of PNPLA3 in liver fat metabolism remains unclear. Indeed, mouse *Pnpla3* knockout models showed no phenotype under a variety of dietary conditions. Specifically, no evidence was found for hepatic steatosis in Role of patatin-like phospholipase domain-containing protein 3 the *Pnpla*3 knockout mice. These observations suggest that PNPLA3 plays no significant physiological role in TG metabolism in mice, but it remains possible that PNPLA3 contributes to hepatic TG metabolism in man. Here, we analyzed *PNPLA* mRNA levels in human and mouse tissues and evaluated the effect of small interfering RNA (siRNA) silencing of PNPLA3 on triglyceride metabolism in human Huh7 and HepG2 hepatoma cells in an effort to elucidate a possible role of PNPLA3 in hepatic TG metabolism.[94-99]

4.4.2 Results

The hepatic expressions of the *PNPLA3*, *PNPLA2* and *PNPLA4* genes were compared in human and mouse liver samples and in the human hepatoma Huh7 cell-line. It was found that

PNPLA2 was prominently expressed in mouse liver, while *PNPLA3* was the highest expressed member of the PNPLA-family in human liver and the human hepatoma Huh7 cell-line. These species specific differences in hepatic expression of PNPLA-family members suggest that it is feasible that human PNPLA3, in contrast to mouse PNPLA3, plays a significant role in hepatic TG metabolism.

In analogy to the physiological role of PNPLA2 in TG-hydrolysis in WAT it can be expected that the putative role of PNPLA3 in hepatic TG-metabolism is related to the TG-hydrolysis activity of PNPLA3. This in turn suggests that PNPLA3 resides in hepatocytes in a subcellular compartment in the vicinity of LDs. It was indeed reported that PNPLA3 was observed near LDs following oleate treatment, while under unstimulated conditions PNPLA3 showed a ubiquitous distribution. We therefore tested this phenomenon in Huh7 cells using confocal microscopy. As shown in Figure 15, (extreme) overexpression of PNPLA3 leads to ubiquitous expression of the protein in the cytoplasm. However, when PNPLA3 is moderately expressed it is localized around LDs. Of note, these studies were performed in the absence of oleate-treatment, indicating that oleate-treatment is not an obligatory requirement for localization of PNPLA3 around LDs.

In order to study the functional role of PNPLA3 we reduced the expression of *PNPLA3* in Huh7 and HepG2 cells using siRNA inhibition and evaluated the effects on TRL secretion and cellular TG content. However, no effects on TRL secretion were observed and no significant changes in cellular TG content were found. Confocal microscopy analyses were performed to validate these observations. As shown in Figure 13, *PNPLA3* siRNA inhibition had no effect on the LD content or size distribution in Huh7 cells, and similar results were obtained in HepG2 cells.

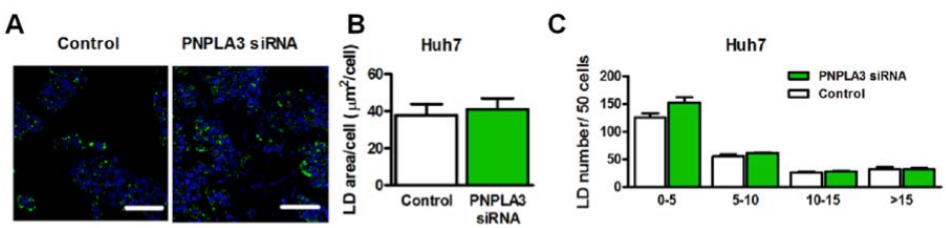

Figure 13. A) Confocal image of control and PNPAL3 siRNA cells showing no visual difference in the amount of LD (in green), which is expressed in B as LD area per cell. There was no difference in the size distribution of the LD particles measured in C. Same experiment was conducted in HepG2 cells with similar results.

We considered the possibility that PNPLA3 influences TG synthesis, not TG hydrolysis as would be expected by comparison with the physiological role of PNPLA2 in white adipose tissue. However, as shown in Figure 14, *PNPLA3* siRNA inhibition had no effect on the

cellular uptake of [^{14}C]palmitate or the incorporation of [^{14}C]palmitate into triglycerides in Huh7 cells, and the same phenomenon was observed in HepG2 cells.

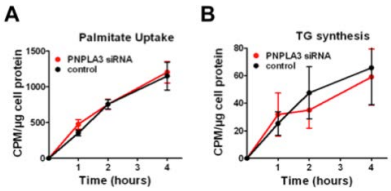

Figure 14. Total cellular [^{14}C]-radioactivity, representing palmitate uptake (A), and triglyceride associated [^{14}C]-radioactivity, representing triglyceride synthesis (B) at 4 time-points. Red line represents the PNPLA3 siRNA samples while the black is the control siRNA.

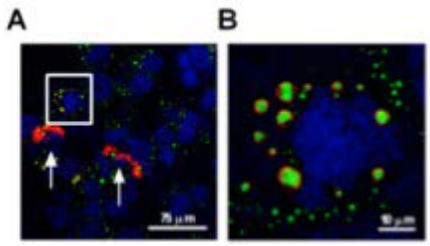

Figure 15. Representative picture of A) PNPLA3-FP635 expression (red), LDs in green. The two Arrows indicate overexpression of PNPLA3. B) magnification of the box in A. Moderate expression of PNPLA3 (red) at the vicinity of the LDs (green). Experiment performed in Huh7 cells.

4.4.3 Discussion

In this study we evaluated the putative role of PNPLA3 in hepatic TG metabolism in human liver and human hepatoma cells. Several lines of evidence pointed to a physiological role of PNPLA3 in hepatic TG metabolism, including a high expression level of *PNPLA3* in human liver and a subcellular localization of PNPLA3 around the LDs in Huh7 cells. However, experimental reductions in PNPLA3 concentrations in human hepatoma cells were not associated with significant changes in the secretion of TRL, with increased cellular accumulation of TGs, or with changes in the rate of TG synthesis. These observations thus provide further evidence against a major physiological role of PNPLA3 in hepatic TG metabolism in man. This provides further support for the hypothesis proposed by Hobbs and coworkers that the PNPLA3 variant I148M represents a gain-of-function mutation leading to excessive accumulation of TGs in the liver in man.

The observed absence of a physiological function of PNPLA3 in hepatic TG metabolism raises the question of the nature of the enzyme responsible for TG-hydrolase activity in human liver. It was recently reported that PNPLA2 is the primary enzyme responsible for TG-hydrolase activity in the mouse liver. It is therefore tempting to speculate that PNPLA2 fulfills a similar role in human liver. However, preliminary experiments from our laboratory found no evidence for a significant contribution of PNPLA2 to hepatic TG-hydrolase activity.

This suggests that an as yet unidentified gene/protein is responsible for TG-hydrolase activity in human hepatocytes. We are currently exploring if other members of the PNPLA-family are involved in this process.[100]

5 CONCLUDING REMARKS

Understanding the pathophysiology of complex diseases is challenging. Unlike monogenic diseases, complex diseases do not follow a linear pattern of inheritance and are caused by multiple genes and their interplay with other genetic, environmental and behavioral factors. Recent GWA studies have uncovered thousands of SNPs associated with complex diseases and intermediary phenotypes. Nevertheless, these SNPs explain thus far only a minor fraction of the total disease-risk or the overall variation in intermediary phenotype. Moreover, the identification of these SNPs only constitutes a first step to uncovering underlying genes and pathways responsible for the observed associations between genotype and disease. In this thesis, I presented studies related to four proteins involved in lipid metabolism, PCSK9, APOE, TM6SF2 and PNPLA3. The results of these studies will help to improve our understanding of the physiological roles of these proteins in hepatic metabolism. At the same time, these studies represent only a small step towards the ultimate goal to provide personalized forms of therapy tailor-made according to the individual's genetic variation.

6 ACKNOWLEDGEMENTS

Low TM6SF2 in the liver → high TG in the liver, low TG secretion into plasma.
High TM6SF2 in the liver → low TG in the liver.

I apologize for the inconvenience. I just had to ☺…

I hardly think anybody other than my opponent, dissertation committee, supervisors (hopefully) and my wife (hopefully^2) will read my full thesis, but I know almost everyone will open this page. 5 years of financing my PhD and this is what it comes down to, a take home message about a gene named after having 6 transmembrane regions when it really has between 7 to 10 and we still really don't know how many ☺. It's hard to purify transmembrane proteins they say. Hopefully, one day TM6SF2 will be given a more deserving name and will be targeted either for reducing plasma triglycerides or for reducing liver fat helping millions. Myself? I do not know where I will be then but I surely know I want to thank many people for these fantastic 5 years!

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